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Owner, by virtue of an invention the title of which is

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(Hebrew) שיבוט מולקולרי של חלבון הקושר TNF

(באנגלית)
(English) Molecular cloning of TNF Binding Protein

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hereby apply for a patent to be granted to me in respect thereof.

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היום 10 בחודש יולי שנת 1990
This 10 of July 1990 of the year

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שיבוט מולקולרי של חלבון הקושר TNF

Molecular cloning of TNF Binding Protein

Yeda Research and Development Co. Ltd.

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Field of the Invention

The present invention relates to Tumor Necrosis Factor (TNF Binding Protein I, herein designated TBPI, and more particularly, to the cloning of the gene coding for said protein and its expression in host cells.

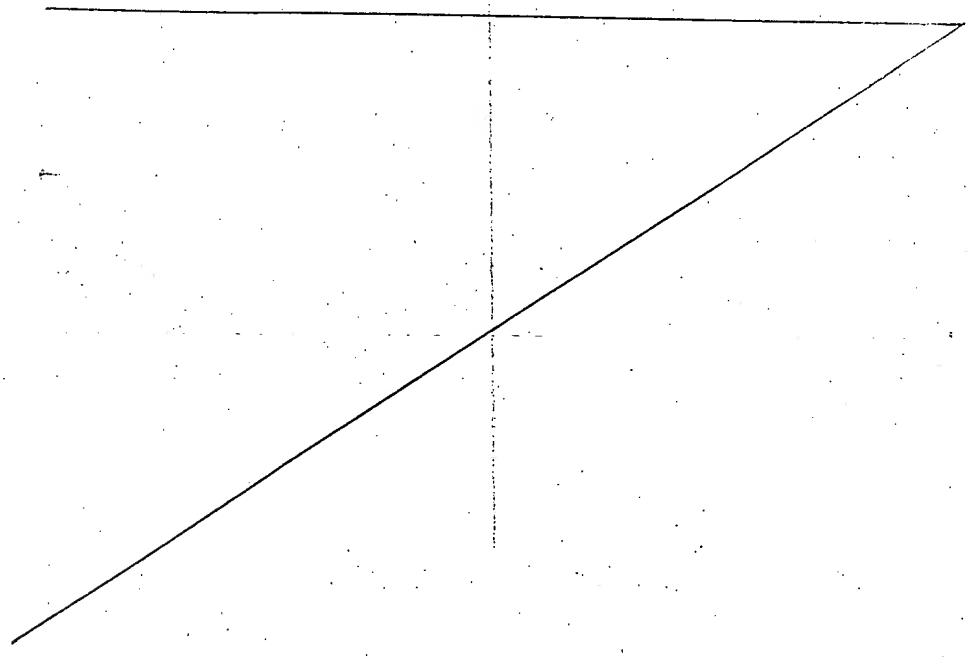
Background of the invention

TNF-α and TNF-β (lymphotoxin) are structurally related polypeptide cytokines, produced primarily by mononuclear leukocytes, whose effects on cell function constitute a major factor in the elicitation of the inflammatory response. The TNFs affect cells in different ways; some of which resemble the functional modes of other inflammatory mediators, like interleukin 1 (IL1) and interleukin 6 (IL6). What appears most distinctive regarding the activity of the TNFs is that many of their effects can result in cell and tissue destruction. Increasing evidence that over-induction of

these destructive activities contributes to the pathogenesis of a number of diseases, makes it of particular interest to elucidate their mechanisms and the ways they are regulated (Old, 1988; Beutler and Cerami, 1988). High affinity receptors, to which both TNF-α and TNF-β bind (Beutler et al., 1985; Kull et al., 1985; Tsujimoto et al., 1985; Baglioni et al., 1985; Aggarwal et al., 1986; Israel et al., 1986) play a key role in the initiation and in the control of the cellular response to these cytokines. These receptors are expressed on the surfaces of a variety of different cells. Studies showing that antibodies reacting with their extracellular portions affect cells in a manner very similar to the TNFs demonstrate that the receptors and cellular components associated with them are sufficient to provide the intracellular signalling for the effects of the TNFs (Espevik et al., 1990). Other studies have shown that molecules related to the TNF receptors (TNF-Rs) exist also in soluble forms. Two immunologically distinct species of such soluble TNF-Rs (TBPI and TBPII) were recently isolated from human urine (Engelmann et al., 1989, 1990b; Olsson et al., 1989; Seckinger et al., 1989a). Immunological evidence indicated that the two proteins are structurally related to two molecular species of the cell surface TNF-R (the type I and type II receptors, respectively). Antibodies to each of the two soluble proteins were shown to block specifically the binding of TNF to one of the two receptors and could be applied to immunoprecipitate the receptors. Antibodies against one of the two soluble proteins (TBPI) were found also

to induce effects characteristic of TNF in cells which express the immunologically cross-reactive cell surface receptors (Engelmann et al., 1990). Like the cell surface receptors for TNF, the soluble forms of these receptors specifically bind TNF and thus can interfere with its binding to cells. It was suggested that they function as physiological inhibitors of TNF activity (Engelmann et al., 1989; Olsson et al., 1989; Seckinger et al., 1989a).

In the present study we explored further the structural relationship of the soluble and cell surface forms of the TNF-Rs by determining amino acid sequences in the soluble forms and by applying amino acid sequence data for one of the soluble receptors to clone the cDNA which encodes this protein. Initial information on the mechanism of formation of the soluble receptors was gained by examining the expression of this cDNA in transfected CHO cells.



Description of the Drawings

Fig. 1. *Nucleotide sequence of the type I TNF receptor cDNA and the predicted amino acid sequence of the encoded protein.*

(A). The probes used for the screening for the cDNA:

a. The NH_2-terminal amino acid sequence of TBPI.

b. Synthetic oligonucleotide probes, designed on the basis of the NH_2-terminal amino acid sequence, used for initial screening.

c. & d. Probes overlapping with the probes presented in b, used to confirm the validity of clones isolated in the initial screening.

B. Schematic presentation of the cDNA clones isolated from a human colon (C2) and from CEM-lymphocytes (E13) libraries and a diagram of the complete cDNA structure. Untranslated sequences are represented by a line. Coding regions are boxed. The shaded portions represent the sequences which encode the signal peptide and the transmembrane domains.

C. Hydropathy profile of the predicted amino acid sequence of the TNF receptor. Hydrophobicity (above the line) and hydrophilicity (below the line) values were determined using the sequence analysis software package of the University of Wisconsin genetic computer group (UWCG) according to Kyte and Doolittle (1982). The curve is the average of the hydrophobicity index for each residue over a window of nine residues.

D. Nucleotide and predicted amino acid sequences of the TNF receptor. The presumptive start and stop signals are denoted by asterisks; the three sequences derived from TBPI by broken overlining; the transmembrane and leader domains by round ended boxes; and the four repetitive sequences in the extracellular domain by thick underlining. Cysteine residues are boxed. Glycosylation sites are overlined and the presumptive polyadenylation signal is underlined.

Fig. 2. *Internal cysteine-rich repeats in the extracellular domain of the TNF-R and their alignment with the homologous repeats in the extracellular domains of human NGF-R and the CDw40 antigen and with sequences of amino acids present in TBPII.*

The amino acid sequences (one-letter symbols) are aligned for maximal homology. Dashes indicate gaps introduced to optimize the alignment. Identities in sequences are shown in boxes. Conservative substitutions (I=L=V; D=E; K=R=H; T=S; G=A; N=Q) are boxed with dotted lines. The positions of the amino acids within the receptors are denoted in the right and left hand margins. The sequences in TBPII (Table I and Materials and Methods) were optimally aligned with the sequences of the other three proteins presented in this figure, using the "Best Fit" alignment program in the UWCG software package.

Fig. 3. *Detection of type I TNF-R using monoclonal antibodies to TBPI in CHO cells transfected with E13 cDNA.*

CHO cells, clones R-18 (transfected with an expression vector in which the E13 cDNA was placed under the control of an SV40 promotor) and C-6 (control; a clone of cells transfected with an expression vector in which E13 was placed in the inverse orientation), and HeLa cells, were stained with the anti TBPI monoclonal antibodies 17, 18, 20 and 30 followed by incubation with FITC conjugated anti mouse F(ab). Fluorescence intensity is compared with that observed when a mouse monoclonal antibody against TNF was used in the first step of the staining as a control.

Fig. 4. *Reversed phase HPLC of the CHO-produced, soluble form of the type I TNF-R.*

A concentrate of the conditioned medium of the CHO R-18 clones (see Fig. 3) and a concentrate of the CHO C-6 clone to which 3 μg pure TBPI was added were applied to an Aquapore RP300 column. Elution was performed with a gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (- - -). Fractions were examined for content of protein (——) and of the soluble form of the type I by an ELISA (▭), as described in Materials and Methods. None of the eluted fractions of a concentrate of the CHO C-6 clone without addition of TBPI was found to contain any detectable amounts of the soluble form of the receptor (not shown).

Fig. 5 *Detection of the mRNA for the type I TNF-R by Northern blotting analysis.*

Hybridization of total RNA (25 μg/lane) from cells of the Jurkat (Gillis and Watson, 1980), CEM (Foley et al., 1965), KG-1 (Koeffer and Golde, 1978), Daudi (Klein and Klein, 1968), K-562 (Lozzio and Lozzio, 1975), U-937 (Sundstroem and Nilsson, 1976), A-673 (Girad et al., 1973), Hep 3B (Aden et al., 1979), HT-29 (Fogh and Trempe, 1975), and HeLa (Gey et al., 1952) lines with the ^{32}P-labelled E13 insert was carried out as described in Materials and Methods. 28s and 18s refer to ribosomal RNA size markers.

Fig. 6. *Involvement of the type I TNF-R in stimulation of protein phosphorylation in cells.*

Effects of TNF-α (1000 u/ml) and rabbit antiserum to TBPI (1:1000) on the phosphorylation of proteins with an MW of 27 kDa in HeLa cells. Untreated cells served as a control. The 27 kDa protein(s) are indicated with an arrow on the right and the migration of markers of molecular weight (Amsham, UK) is shown on the left. Normal rabbit serum had no effect at a dilution of 1:1000 (not shown).

Fig. 7. *Time course of the release of COOH terminal amino acids from TBPI by carboxypeptidase Y.*

Summary of the Invention

Purified TBP-I isolated from human urine was described in our Patent Application No. 88378 and shown to contain at the N-terminus the amino acid sequence shown in Fig. 1A a.

The invention relates to oligonucleotide probes to the cDNA coding for a protein comprising the amino acid sequence of TBP-1. The probes were synthesized by known methods on the basis of the above amino acid sequence of the N-terminus of TBP-I.

The invention also relates to a DNA molecule comprising a recombinant DNA molecule or a cDNA molecule coding for a protein comprising the amino acid sequence of TBP-I or a protein substantially homologous therewith. Within the scope of the invention are DNA molecules encoding said homologous proteins having the same biological activities of TBP-I. In a preferred embodiment, the DNA molecule is a cDNA molecule picked up from a human cDNA library, in particular a colon cDNA library.

The invention further comprises cloning of said cDNA molecule into a replicable plasmid vector and transformation of a bacterium, e.g., competent E.coli TG1 therewith.

In another aspect, the invention comprises the isolation of mRNA coding for a protein comprising the amino acid sequence

of TBP-I by extraction from cells and its detection by hybridization with the cDNA of the invention.

Once the mRNA is obtained in a purified form, the cDNA coding for a protein comprising the amino acid sequence of TBP-I can be obtained by contacting the mRNA with reverse transcriptase for a time and under conditions sufficient to form said cDNA. This cDNA may be converted to double stranded cDNA by known techniques.

Probes may be prepared from the cDNA sequences of the invention and used for isolation of the genomic DNA coding for a protein comprising the amino acid sequence of TBP-I by known methods.

The DNA of positive clones are then inserted into appropriately constructed expression vectors by techniques well known in the art.

In order to be capable of expressing a desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. The gene must be preceded by a promoter in order to be transcribed. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

The DNA molecule comprising the nucleotide sequence coding for a protein comprising the amino acid sequence of TBP-I preceded by a nucleotide sequence of a signal peptide and the operably linked transcriptional and translational regulatory signals is inserted into a vector which is capable of integrating the desired gene sequences into the host cell chromosome. The cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector.

In a preferred embodiment, the introduced DNA molecule will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, etc.

Host cells to be used in this invention may be either prokaryotic or eukaryotic. Preferred prokaryotic hosts include bacteria, such as E.coli. Under such conditions, the protein will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

Preferred eukaryotic hosts are mammalian cells, e.g., human, monkey, mouse and chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Also yeast and insect cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast.

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired protein or a fragment thereof. The expressed protein is then isolated and purified by any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like.

In a preferred embodiment, CHO cells are transfected with th type I TNF-R cDNA of the invention and they produce both th cell surface receptor and TBPI, its soluble form.

The invention will be illustrated by the following examples:

Materials and methods

Determination of amino acid sequences within the TNF-binding proteins TBPI and TBPII

The TNF binding proteins TBPI and TBPII were isolated from concentrated preparations of urinary proteins, as described previously (Engelmann et al., 1990) by ligand (TNF) affinity chromatography followed by reversed phase HPLC. TBPI was cleaved with cyanogen bromide, yielding two peptides which, following reduction and alkylation (Andrews and Dixon, 1987), were isolated by reversed phase HPLC. The two peptides (CNBr-1 and CNBr-2 in Table I) were subjected to NH_2-terminal sequence analysis on a pulsed liquid gas phase protein microsequencer (Model 475A, Applied Biosystems Inc., Foster City, CA). The sequence found for one of the peptides (CNBr-1) was identical to the NH_2 terminal sequence of the intact TBPI protein (Engelmann et al., 1989, 1990).

The COOH terminal sequence of amino acids in TBPI was determined by digestion of the protein with carboxypeptidase Y followed by sequential analysis of the released amino acids. A sample of pure TBPI (32 μg) was mixed with 1 nmole of norleucine, as an internal standard, dried thoroughly and resuspended in 8 μl 0.1 M sodium acetate buffer, pH 5.5, containing 0.8 μg carboxypeptidase Y (Sigma, St. Louis, MO). Digestion was performed at room temperature. 2 μl aliquots withdrawn at various time points were acidified by adding 3 μl of 10% acetic acid to each, followed by addition of 15 μl 0.5% EDTA. They were then subjected to automated amino acid analysis (Applied Biosystems, U.K., mod. 420A). The results (Fig. 7) indicate the sequence -Ile-Glu-Asn-COOH.

Sequences within TBPII were determined by generation of tryptic peptides of the protein. A sample of pure TBPII (200 μg) was reduced, alkylated and repurified on an Aquapore RP-300 reversed phase HPLC column. Fractions containing the modified protein were pooled and the pH was adjusted to 8.0 with $NaHCO_3$. Digestion with TPCK-trypsin (238 u/mg, Millipore Corp., Freehold, NJ) was performed for 16 h at room temperature at an enzyme to substrate ratio of 1:20 (w/w). The digest was loaded on a C_{18} RP-P reversed phase HPLC column (Synchrom, Linden, IN) and the peptides separated by a linear 0 to 40% acetonitrile gradient in 0.3% aqueous trifluoroacetic acid. The NH_2 terminal amino acid sequences of the peptides and of the intact protein (N-terminus) are presented in Table I. The peptides

were numbered according to their sequence of elution from the RP-P column. In the fractions denoted as 39, 44, 46, 53 and 54, where heterogeneity of sequences was observed, both the major and the secondary sequences are presented.

Isolation of cDNA clones

Three mixtures of synthetic oligonucleotide probes generated from the nucleotide sequence deduced from the NH_2-terminal amino acid sequence of TBPI were used for the screening of cDNA libraries. Initial screenings were carried out with 48-fold degenerated, 26-mer in which deoxyinosine was introduced, wherever the codon ambiguity allowed for all four nucleotides (Fig. 1Ab). The validity of positive clones was examined by testing their hybridization to two mixed 17-mer nucleotide sequences containing 96 and 128 degeneracies, corresponding to two overlapping amino acid sequences which constitute part of the sequences to which the 26-base probes correspond (Fig. 1Ac and d). An oligonucleotide probe corresponding to a sequence located close to the 5′ terminus of the longest of the partial cDNA clones isolated with the degenerated probes (Nucleotides 478–458 in Fig. 1D) was applied for further screening cDNA libraries for a full length cDNA clone. ^{32}P-labeling of the probes, using T4 polynucleotide kinase, plating of the phages in lawns of bacteria, their screening with the radiolabelled probes, isolation of the positive clones,

and subcloning of their cDNA inserts were carried out using standard procedures (Sambrook et al., 1989).

Nucleotide sequencing of the cDNA clones

cDNA inserts isolated from positive λGT11 recombinant phages were subcloned into the pBluescript KS(-) vector. Inserts found in λZAP phages were rescued by excising the phasmid pBluescript SK(-) in them, using the R408 helper phage (Short et al., 1988). DNA sequencing in both directions was done by the dideoxy chain termination method (Sanger et al., 1977). Overlapping deletion clones of the cDNAs were generated, in both orientations, by digestion of the cDNA with exonuclease III ("Erase a base" kit, Promega Biotec, Madison, WI). Single stranded templates derived from these clones using the R408 phage were sequenced with a T7 DNA polymerase sequencing system (Promega).

Constitutive expression of the type I human TNF-R in CHO cells

The E13 insert was introduced into a modified version of the pSVL expression vector. This construct was transfected, together with the pSV2-DHFR plasmid which contains the DHFR cDNA, into DHFR deficient CHO cells (Chernajovsky et al., 1984), using the calcium phosphate precipitation method (Chen and Okayoma, 1987). Transfection with a recombinant pSVL vector which contained the E13 insert in the inverse orientation

served as a control. Cells expressing the DHFR gene were selected by growth in nucleotide-free MEM alpha medium containing fetal calf serum which had been dialyzed against phosphate buffered saline. Individual clones were picked out and then further selected for amplification of the transfected cDNAs by growth in the presence of 500 nM sodium methotrexate.

Detection of surface-expressed type I TNF-R in the CHO cells

Binding of radiolabelled human rTNF to cells (seeded in 15 mm tissue culture plates at a density of 2.5×10^5 cells/plate) was quantitated as described before (Holtmann and Wallach, 1987).

To examine the binding of monoclonal antibodies against TBPI to the CHO cells the cells were detached by incubation in phosphate buffered saline (PBS: 140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$), containing 5 mM EDTA and then incubated for 45 min at 0°C with 50 μg/ml of the test monoclonal antibody in PBS containing 0.5% bovine serum albumin, and 15 mM sodium azide (PBS/BSA). After washing the cells with PBS/BSA they were incubated further for 30 min at 0°with FITC labelled, affinity purified goat antibody to the F(ab) fragment of mouse IgG (1:20 in PBS/BSA) (Bio-Makor, Israel) and then analyzed by determining the intensity of fluorescence in samples of 10^4 cells using the Becton Dickinson fluorescence activated cell sorter 440. Four monoclonal

antibodies to TBPI, clones 17, 18, 20 and 30, shown by cross competition analysis to recognize four spatially distinct epitopes in the TBPI molecule (Pat. Appln. No. 94039) and, as a control, a monoclonal antibody against TNF-α (all purified from ascitic fluids by ammonium sulphate precipitation and of the IgG_2 isotype) were used.

Quantitation of the soluble form of the type I TNF-R by ELISA

A sensitive enzyme linked immunosorbent assay was set up, using TBPI-specific monoclonal and polyclonal antibodies in a sandwich technique. Immunoglobulins of the anti-TBPI mAb clone 20 (Pat. Appln. No. 94039) were adsorbed to 96 well ELISA plates (maxisorp, Nunc, Denmark) by incubation of the plates for 2 h at 37°C with a solution of 25 μg/ml of the antibody in PBS. After incubating the wells further for 2 h at 37°C with a solution containing phosphate buffered saline, 1% BSA, 0.02% NaN_3 and 0.05% Tween 20 (blocking solution) to block nonspecific further binding of protein, tested samples were applied in aliquots of 50 μl/well. The plates were then incubated for 2 h at 37°C, rinsed 3 times with PBS supplemented with 0.05% Tween 20 (washing solution) and rabbit polyclonal antiserum against TBPI, diluted 1:500 in blocking solution, was then added to the wells. After further incubation for 12 h at 4°C the plates were rinsed again and incubated for 2 h with horse raddish peroxidase-conjugated purified goat anti rabbit IgG. The assay was

developed using 2,2′-azino-bis (3-ethylbenz thiazoline-6 sulfonic acid) as a substrate (Sigma). The enzymatic product was determined colorimetrically at 600 nm. Pure TBPI served as a standard.

Detection of a soluble form of the type I TNF-R in the growth medium of the transfected CHO cells and its analysis by reversed phase HPLC

The amounts of soluble form of the type I TNF-R in samples of the growth medium of the tested CHO cells, collected 48 h after medium replacement, were determined by the immunoassay described above. For analysis of the soluble receptor by reversed phase HPLC the CHO cells were cultured for 48 h in serum-free medium (nucleotide-free MEM α). The medium samples were concentrated 100-fold by ultrafiltration on an Amicon PM5 membrane and 100 μl aliquots were then applied to an Aquapore RP300 column (4.6 × 30 mm, Brownlee Labs) preequilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with this solution at a flow rate of 0.5 ml/min until all unbound proteins were removed, and then eluted with a gradient of acetonitrile concentration in 0.3% aqueous trifluoroacetic acid, as described before (Engelmann et al., 1989). Fractions of 0.5 ml were collected and, after concentration in vacuo, were neutralized with 1 M HEPES buffer pH 9.0. Amounts of soluble type I TNF-R in the fractions were determined by ELISA and the concentration of protein by the fluorescamine method (Stein and Moschera, 1981).

RNA isolation and analysis

RNA was isolated by a modification of the procedure described by Feramisco et al. (Feramisco et al., 1982; Queen and Baltimore, 1983) and analyzed by electrophoresis in 1.5% agarose/6% formaldehyde gels, followed by blotting to "Gene screen plus" hybridization transfer membranes (NEN, Boston, MA). The E13 cDNA insert was ^{32}P labelled by random oligomer priming, using the Amersham random primer labelling kit (Amersham, UK). The membranes were hybridized at 42°C in the presence of 50% formamide and then washed as prescribed by Sambrook et al. (1989) for the detection of low abundance sequences.

Determination of the effect of TNF and of antibodies to TBPI on protein phosphorylation

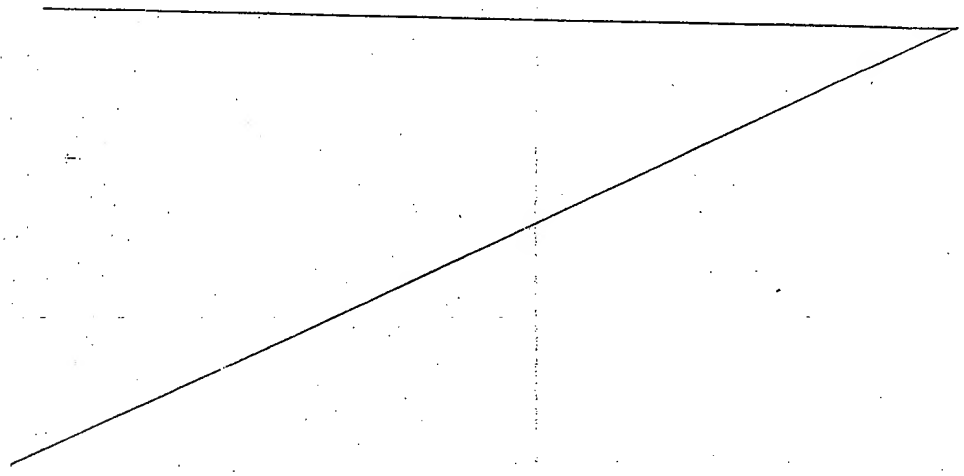
Confluent monolayers of HeLa cells (Gey et al., 1952), in 9 mm microwells, were incubated for 100 min with 100 μCi/ml [^{32}P] orthophosphate (Nuclear Research Center, Beer Sheva, Israel) in phosphate-free DMEM containing 10% fetal calf serum which had been dialyzed against 0.9% NaCl. Recombinant human TNF-α and rabbit antiserum to TBPI (1:1000) (Engelmann et al., 1990) or, for comparison, normal rabbit serum (1:1000) were then added to the cells for 20 min. The cells were

rinsed, and immediately solubilized by boiling in sample buffer for SDS-PAGE containing β-Mercaptoethanol. The solubilized proteins were analyzed by SDS-PAGE (12%) followed by autoradiography.

Example 1

Cloning of the cDNA for the Type I TNF-R

To clone the cDNA which codes for the TNF-binding protein, TBPI, and its related TNF receptor, we screened several cDNA libraries, using 3 overlapping oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBPI (Fig. 1A). In a λGT11 library derived from the mRNA of human colon (randomly primed, Clontech, Palo Alto, CA), we detected four recombinant



phages which hybridized with the three probes. The inserts in those four phages were similar in size, and in restriction mapping and sequence analysis were found to overlap. Complete analysis of the sequence of the longest of the four (C2 in Fig. 1B, deposited on 6.12.1989 with the CNCM, Paris,France, No I-917) revealed an open reading frame, extending over its entire length. A polypeptide chain encoded in this reading frame fully matches the NH_2-terminal amino acid sequence of TBPI. Neither an initiation nor a stop codon was found in the C2 insert. Rescreening the colon cDNA library, using another probe corresponding to a sequence found in C2 (see Materials and Methods), yielded several other recombinant phages containing inserts that overlap with the C2 insert. However, none of them provided further sequence information on the cDNA in the 5′ or the 3′ direction. In a λZAP cDNA library derived from the mRNA of CEM lymphocytes (Foley et al., 1965) (oligo dT and randomly primed, Clontech) 5 phages hybridizing with this probe were detected, containing significantly longer inserts than C2. The longest insert (E13, Fig. 1B) was sequenced in its entirety (Fig. 1D) and was found to contain the C2 sequence (nucleotides 346–1277 in Fig. 1D) within one long open reading frame of 1365 bp, flanked by untranslated regions of 255 and 555 nucleotides at its 5′ and 3′ ends respectively. The potential ATG initiation site, occurring at positions 256–258 in the nucleotide sequence, (denoted by an asterisk in Fig. 1D) is preceeded by an upstream in-frame termination codon at bases 244–246. The start location

is in conformity with one of the possible alternatives for the translation initiation consensus sequence (GGCATGG, nucleotides 253–259, Kozak, 1987).

There is no characteristic poly(A) addition signal near the 3′ end of the cDNA. The sequence ACTAAA, at nucleotides 2044–2049, may serve as an alternative to this signal, but with low efficiency (Sheets and Wickens, 1989). At nucleotides 1964–1999, there is a consecutive six-fold repetition of the sequence G(T)n (n varying between 4 and 8). Similar sequences have been observed also in the 3′ noncoding regions of the cDNAs of some members of the *jun* family, which are also devoid of the characteristic poly(A) signal (Ryder et al., 1988, 1989). The 3′ end has a 15 base poly (A) tail.

The size of the protein encoded by the cDNA (about 50 kD) is significantly larger than that of TBPI (Engelmann et al., 1989; Olsson et al., 1989; Seckinger et al., 1989a). A hydropathy index computation (Kyte and Doolittle, 1982) of the deduced amino acid sequence of the protein (Fig. 1C) revealed two major hydrophobic regions (see round-ended boxes in Fig. 1D). One, at its NH_2-terminus, is apparently the signal peptide whose most likely cleavage site (Von Heijne, 1986) lies between the glycine and isoleucine residues designated in Fig. 1D as -1 and $+1$ respectively. The other major hydrophobic domain, located between residues 191 and 213, is flanked at both ends by several charged amino acids, characteristic of a membrane anchoring domain (Pidgeon et al., 1989). As in several other transmembrane proteins, the

amino acids confining the hydrophobic domain at its COOH terminal are basic. The transmembrane domain bisects the predicted protein into nearly equivalent sized extracellular and intracellular domains.

The extracellular domain has 3 putative sites for asparagine-linked glycosylation (overlined in Fig. 1D). Assuming that the amount of oligosaccharides in the extracellular domain is similar to that reported as present in TBPI (Seckinger et al., 1989b), the molecular size of the mature protein is very similar to that estimated for the type I receptor (about 58 kD) (Hohmann et al., 1989).

Features of the predicted amino acid sequence in the type I TNF-R and relationship to the structure of TBPI and TBPII

The amino acid sequence in the extracellular domain of the protein encoded by the E13 cDNA fully matches several sequences of amino acids determined in TBPI (Table I). It contains the NH_2-terminal amino acid sequence of TBPI at amino acids 20–32 (compare Fig. 1D and Fig. 1Aa), a sequence corresponding to the COOH terminus of TBPI at amino acid 178–180; and, adjacent to the first methionine located further downstream in the encoded protein, also a sequence which is identical to the NH_2-terminal amino acid sequence of a cyanogen-bromide cleavage fragment of TBPI (broken lines in Fig. 1D). There is also a marked similarity in amino acid composition between the extracellular domain of the receptor and TBPI (Table II).

The most salient feature of this amino acid composition is a very high content of cysteine residues (shown boxed in Fig. 1D). The positioning of the cysteine residues as well as of other amino acids within the extracellular domain displays a four-fold repetition pattern (Fig. 2 and underlined in Fig. 1D). As shown in Fig. 2, there is a marked homology between this four-domain structure and sequences found in the extracellular domains of the receptor for the nerve growth factor (NGF-R) (Johnson et al., 1986; Radeke et al., 1987) and, to a somewhat lower degree, also to sequences in the extracellular domain of the recently cloned receptor-like B cell antigen CDw40 (Braesch-Andersen et al., 1989; Stamenkovic et al., 1989). The amino acid sequence within the extracellular domain of the TNF-R, which corresponds to the COOH terminal sequence of TBPI (see Table I and Fig. 7), is located at the COOH terminus of the cysteine-rich repeat region. The sequence corresponding to the NH_2 terminal sequence of TBPI corresponds to a sequence located a few amino acids upstream of the NH_2 terminal end of this region (broken lines in Fig. 1D) in the extracellular domain

In contrast to the identity of amino acid sequences between TBPI and the extracellular domain of the type I receptor, sequences examined in the soluble form of the type II TNF-R (TBPII, Table I) were not identical with any sequence in the type I TNF-R. This finding is expected, considering the lack of immunological crossreactivity between the two receptors (Engelmann et al., 1990). However,

as demonstrated in Fig. 2, the sequences in TBPII have a significant homology of structure with the four-fold cysteine-rich repeat region in the extracellular domains of the type I TNF-R, the NGF-R and the CDw40 protein. The similarity between TBPII and the CDw40 protein is particularly notable and seems to extend also to a region within the two proteins which protrudes from the cysteine-rich structures in the NH_2 direction (Fig. 2).

In contrast to the very high content of cysteine residues in the putative extracellular domain of the type I TNF-R, there are only 5 cysteine residues in the intracellular domain. Between the two which are proximal to the transmembrane domain (positions 227 and 283), extends a stretch of 55 amino acids which is rich in proline residues (16% of the residues) and even richer in serine and threonine residues (36%), most located very close to or adjacent to each other. The consensus sequence Gly-x-Gly-x-x-Gly which characterizes protein kinases and various other nucleotide binding proteins (Kamps et al., 1984) is not present in the intracellular domain.

Example 2

Expression of the type I TNF-R cDNA

To further explore the relation between the protein encoded by the E13 cDNA and TBPI, we expressed this protein in CHO cells. The E13 cDNA was introduced

into an expression vector and was cotransfected with a recombinant vector containing the DHFR cDNA into DHFR-deficient cells. After selection by growth in a nucleotide-free medium, individual clones were amplified by growth in the presence of methotrexate. A number of clones which react with several monoclonal antibodies that bind to spatially distinct epitopes in TBPI were detected (Fig. 3). Expression of the protein was correlated with an increase in specific binding of human TNF to the cells (Table III).

Applying a sensitive immunoassay for TBPI, in which polyclonal antibodies and a monoclonal antibody against this protein were employed, we could detect in the growth medium of CHO cells which express the human TNF-R on their surface also a soluble form of the protein (Table III). All of five different CHO clones which expressed the TNF-R, produced this soluble protein. Several other transfected clones which did not express the cell surface receptor did not produce its soluble form either (not shown). When analyzed by reversed phase HPLC, the CHO-produced soluble TNF-R eluted as a single peak, with a retention time identical to TBPI (Fig. 4).

Example 3

Northern blot analysis using the E13 cDNA as a probe

To gain information on the transcripts which encode the type I TNF-R, we tested the mRNA of cells of differing origin for their ability to hybridize with the E13 cDNA. As shown in Fig. 5, in all the cell types, including the HT29 cells, which

secrete continuously a soluble form of the type I TNF-R,

only a single hybridizing transcript was detectable, in all cases of the same size — about 2300 bp, corresponding to the full length of the cDNA. Interestingly, significant amounts of this type I TNF-R mRNA could be detected also in the U937 cells, in which the prevalent TNF-R is type II (Engelmann et al., 1990), suggesting that posttranscriptional mechanisms take part in the control of the expression of the type I receptor.

Example 4

Evidence for the involvement of the type I TNF-R in the stimulation of protein phosphorylation by TNF

Treating cells with TNF results in a rapid increase in the phosphorylation of certain specific cellular proteins including some with a MW of about 27 kDa (Hepburn et al., 1988; Kaur and Saklatvala, 1988; Schutze et al.,1989). Since it is apparent from the sequence data of the intracellular domain of the type I TNF-R that this receptor is devoid of intrinsic protein kinase activity, it was of interest to examine the extent to which this receptor is involved in TNF-mediated protein phosphorylation events. Antibodies to TBPI induce various effects in cells which are characteristic of TNF. This activity was shown to be correlated with the ability of the antibodies to cross-link the type I TNF-R molecules. As shown in Fig. 6, treating HeLa cells which express the type I TNF-R (Engelmann et al.,

1990) with antibodies to TBPI induced, as does TNF, a marked increase in the phosphorylation of protein(s) with a MW of 27 kDa, confirming that the type I TNF-R is involved in this effect.

Discussion

There is accumulating evidence for the natural occurrence of soluble forms of cell surface receptors. Such forms have been identified for example for the receptor to interleukin-2 (IL-2) (Rubin et al., 1985; Osawa et al., 1986), growth hormone (Leung et al., 1987), NGF (DiStefano and Johnson, 1988), interleukin-6 (Novick et al., 1989) interferon-γ (Novick et al., 1989) and tumor necrosis factor (Engelmann et al., 1989; 1990 ; Olsson et al., 1989; Seckinger et al., 1989a). Yet knowledge of the exact structure of these soluble receptors and of the mechanisms of their formation is still limited. Most thoroughly characterized, so far, is the soluble form of the 55 kDa receptor for IL-2. Based on detailed sequence analysis and studies of its mode of formation in cultured cells, it was suggested that it is derived from the cell surface form of the receptor by proteolytic cleavage (Robb and Kutny, 1987).

A different mechanism for the formation of soluble receptors was proposed in two recent studies describing the cloning of the cDNAs for the receptors to IL-4 and IL-7. Besides cDNA clones encoding the full length receptors, also clones which encode truncated, soluble forms of these receptors were isolated in these studies. It was

suggested that these latter clones are derived from transcripts specifically encoding soluble forms of the receptors, transcribed from the same genes which encode the cell surface forms, but differently spliced (Mosley et al., 1989; Goodwin et al., 1990).

Data presented in our study are consistent with the notion that TBPI — the soluble form for the type I TNF-R — constitutes a fragment of the cell surface form of this receptor, corresponding to its extracellular domain. The receptor is recognized by several monoclonal antibodies to TBPI which interact with several spatially distinct epitopes in this protein (the present study and Engelmann et al., 1990a). The amino acid sequence in the extracellular domain fully matches several sequences present in TBPI. Furthermore, the amino acid composition of the region within the extracellular domain which extends between those residues which correspond to the NH_2 and COOH termini of TBPI, is very similar to the amino acid composition reported for TBPI. There is also a similarity in size between TBPI and this part of the receptor (taking into account that about a third of the TBPI molecule consists of oligosaccharides (Seckinger et al., 1989b)). Particularly informative with regard to the mechanism of formation of TBPI is the finding that a soluble form of the type I TNF-R is produced by CHO cells which were transfected with the TNF-R cDNA. This finding implies that cells possess some mechanism(s) which allow(s) the formation of the soluble form of the TNF-R from that same transcript which encodes the cell surface form. There is no indication from the data

of this study for the existence of transcripts which encode specifically soluble forms of TNF-R. Northern blot analysis did not reveal transcripts smaller than the full size of the TNF-R mRNA in any of the cells examined, not even in the HT29 cells, which continuously release into the culture medium significant amounts of a soluble form of the type I TNF-R. Furthermore, sequence and restriction mapping analyses of the various cDNA clones isolated in this study together with the C2 and E13 clones failed to reveal any difference in structure, besides differences in size, between these clones and the E13 cDNA (data not shown). The amino acid sequence data of TBPI also provide no indication of the existence of transcripts specific to this protein. Soluble receptors produced from alternatively spliced transcripts, as suggested for the IL-4 and IL-7 receptors, are expected to have unique COOH terminal sequences (Mosley et al., 1989; Goodwin et al., 1990). The COOH terminal sequence of TBPI was found to be identical to a sequence found in the cell surface receptor. Still, existence of a minor population of transcripts which specifically code for soluble forms of TNF-Rs, at amounts lower than the limit of detection by the techniques employed, although not supported by the data presented in this study, cannot be excluded.

The low rate of production of the soluble form of the type I TNF-R by the E13-transfected CHO cells does not necessarily reflect maximal activity. In HT29 cells, the spontaneous release of a soluble form of type I TNF-R occurs at about a 10-fold higher rate than that observed with the CHO-R-18 clone (data not shown).

Furthermore, a recent study (Porter and Nathan, 1990) indicates that the mechanism of formation of the soluble TNF-R can be effectively enhanced by certain specific stimuli. Stimulation of human neutrophils with FMLP, or with several other physiological stimuli, was found to result, within a few minutes, in an extensive decrease of the cell-surface expressed TNF-R and an accompanying release of a soluble form of these receptors, similar in size to TBPI. A likely mechanism whereby soluble forms of TNF receptors can be derived from the same transcripts which encode the cell surface forms is proteolytic cleavage. Indeed, flanking the amino acid residue which corresponds to the NH_2-terminus of TBPI there are, within the sequence of amino acids of the receptor, two basic amino acid residues (Lys-Arg) which can serve as a site of cleavage by trypsin-like proteases. The identity of the proteases which might cause cleavage to take place at the COOH terminus of TBPI is not known. In view of the marked structural homology between the extracellular domain of the type I TNF-R and the soluble form of the type II TNF-R (TBPII) as well as the homology with the extracellular domain of the NGF-R, for which existence of a soluble form has been also documented (DiStefano and Johnson, 1988; Zupan et al., 1989), it is tempting to speculate that a common mechanism of cleavage and similar cleavage sites are involved in the formation of the soluble forms of those three receptors.

Table I: Amino acid sequences of TBP I and TBP II

TBP I:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
CNBr-1 (=N-terminus)	NH_2	Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	Tyr	ILe	His	Pro	Gln	---	
CNBr-2	NH_2	Gly	Gln	Val	Glu	ILe	Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	---
C-terminus	---	ILe	Glu	Asn	COOH											

TBP II:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
N-terminus	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg	Leu	Arg	Glu	Tyr	Tyr	---
TRP 35	NH_2	Leu	Cys	Ala	Pro	Leu	Arg	Lys																
TRP 39/1	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 39/2	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---											
TRP 44/1	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	Ser	---										
TRP 44/2	NH_2	Ser	Cys	Gly	Pro	Ser	Tyr	Pro	Asp	---														
TRP 46/1	NH_2	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg										
TRP 46/2	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 50	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 54/1	NH_2	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	Lys														
TRP 54/2	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg						
TRP 53/1	NH_2	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg								
TRP 53/2	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 60	NH_2	Ile	Cys	Thr	Cys	Arg	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	---									
TRP 62	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	Lys			
TRP 65	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	Lys			
TRP 67	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys			
TRP 84	NH_2	Thr	Ser	Asp	Thr	Val	Cys	Asp	Ser	Cys	Glu	Asp	Ser	Thr	Tyr	Thr	Gln	Leu	Trp	---				

Table II. *Similarity of the amino acid compositions of the TNF binding protein TBPI and a corresponding region in the extracellular domain of the TNF-R (type I)*

Amino acid	mol/100 mol of amino acids	
	TBPI*	Residues 20–180 in the extracellular domain**
Ala 1.7	1.2	
Cys	12.8	14.9
Asp + Asn	10.0	11.1
Glu + Gln	13.9	12.4
Phe	3.2	3.1
Gly	6.3	5.6
His	4.4	4.3
Ile	2.8	2.5
Lys	6.2	6.2
Leu	8.0	6.8
Met	0.4	0.6
Pro	3.8	3.1
Arg	4.7	4.3
Ser	8.1	9.3
Thr	6.1	6.2
Val	4.2	4.3
Trp	–	0.6
Tyr	2.4	3.1

* According to Olsson et al., 1989

** Residue 20 corresponds to the NH_2-terminal amino acid of TBPI. Residue 180 is the COOH-terminal residue of TBPI.

Table III. *Expression of the cell surface and soluble forms of human type I TNF-R in CHO cells*

CHO cell clone	Specific binding of TNF (CPM/10^6 cells)	cells expressing human cell surface TNF-R (% fluorescent cells)	human soluble type I TNF receptors (pg/ml)
nontransfected	180±45	<1%	<0.03
C6	175±50	<1%	<0.03
R-16	550±60	73%	30
R-18	610±40	89%	49

The R-16 and R-18 clones consist of cells transfected with a recombinant expression vector containing E13 cDNA. C-6 cells were transfected with a control vector (see Fig. 3). Binding of radiolabelled TNF to the cells was determined in pentaplicate samples. Detection of immunoreactive receptors on the surface of the cells was carried out using combined 17, 18, 20 and 30 anti TBPI monoclonal antibodies. Results are expressed as percentage of fluorescent cells (background values, obtained by staining the cells with an anti-TNF monoclonal antibody, are subtracted). For other details, see Materials and Methods.

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Claims

1. A DNA molecule comprising a recombinant DNA molecule or a cDNA molecule coding for a protein comprising the amino acid sequence of TBPI or a protein substantially homologous therewith.

2. A DNA molecule comprising the E13 insert represented by the nucleotide sequence shown in Figure 1D.

3. A cDNA molecule according to claim 1 comprising the C2 insert represented by the nucleotide sequence 346-1277 shown in Figure 1D or a nucloetide sequence substantially homologous therewith.

4. A cDNA molecule according to claim 1 comprising the nucleotide sequence 376-858 shown in Figure 1D or a nucleotide sequence substantially homologous therewith.

5. Oligonucleotide probes useful for picking up genes from cDNA libraries which code for proteins comprising the amino acid sequence of TBPI, said probes having the formulae shown in Figures 1A b, 1A c and 1A d.

6. DNA molecules hybridizable to all three oligonucleotide probes according to claim 5 and which code for a protein comprising the amino acid sequence of TBPI or a protein substantially homologous therewith.

7. A replicable expression vector comprising a DNA molecule according to any of claims 1 to 4.

8. A replicable expression vector according to claim 7 comprising the E13 insert of claim 2.

9. A host cell transformed with a replicable expression vector according to claim 7 or 8.

10. A host cell according to claim 9 which is a prokaryotic cell.

11. A host cell according to claim 9 which is a eukaryotic cell.

12. A chinese hamster ovary cell according to claim 11 which is transformed with a replicable expression vector according to claim 8.

13. A method for the production of recombinant TBPI comprising culturing the cells according to claim 12 and recovering the TBPI thus produced.

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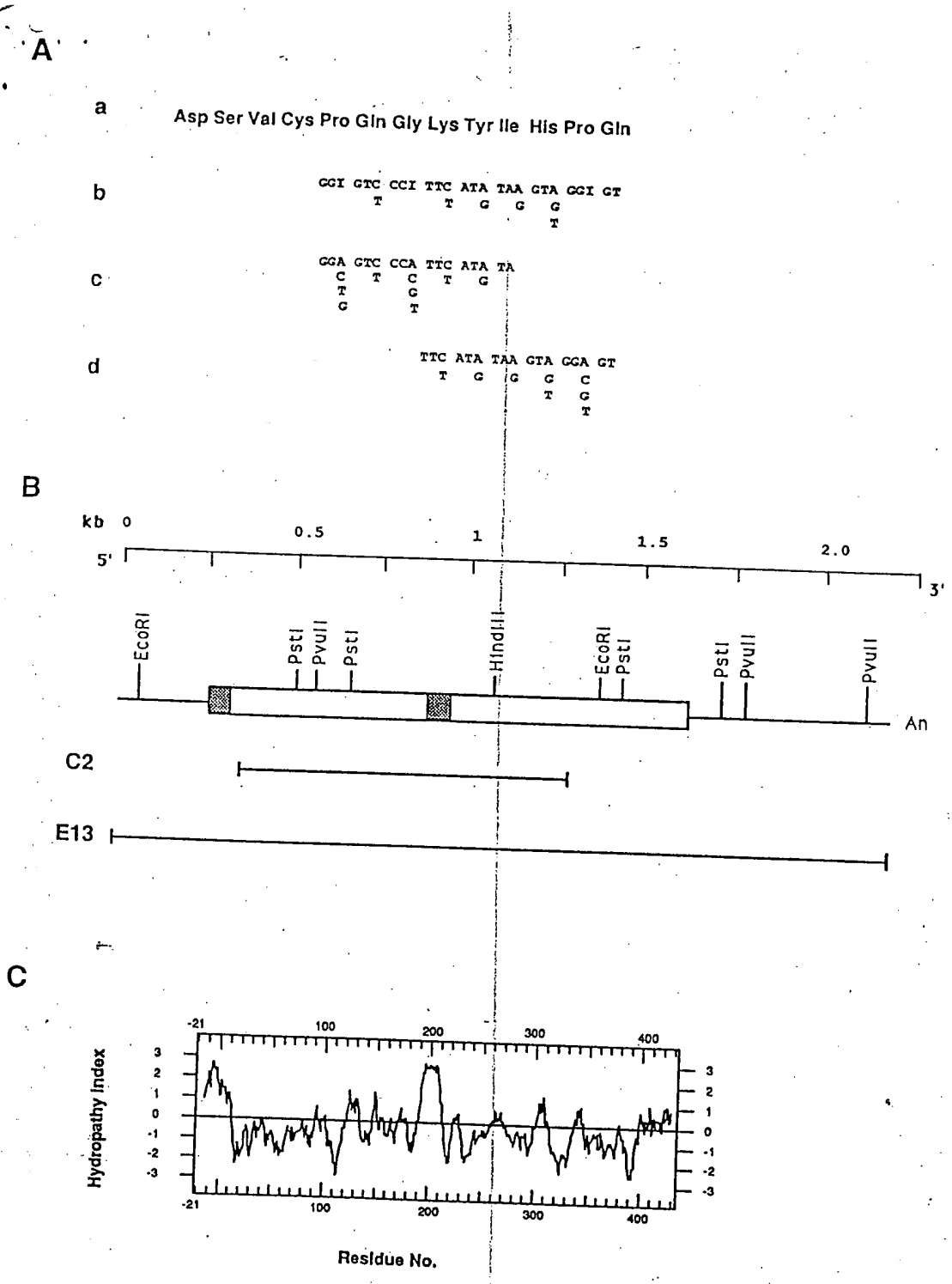

FIGURE 1 A-C

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D

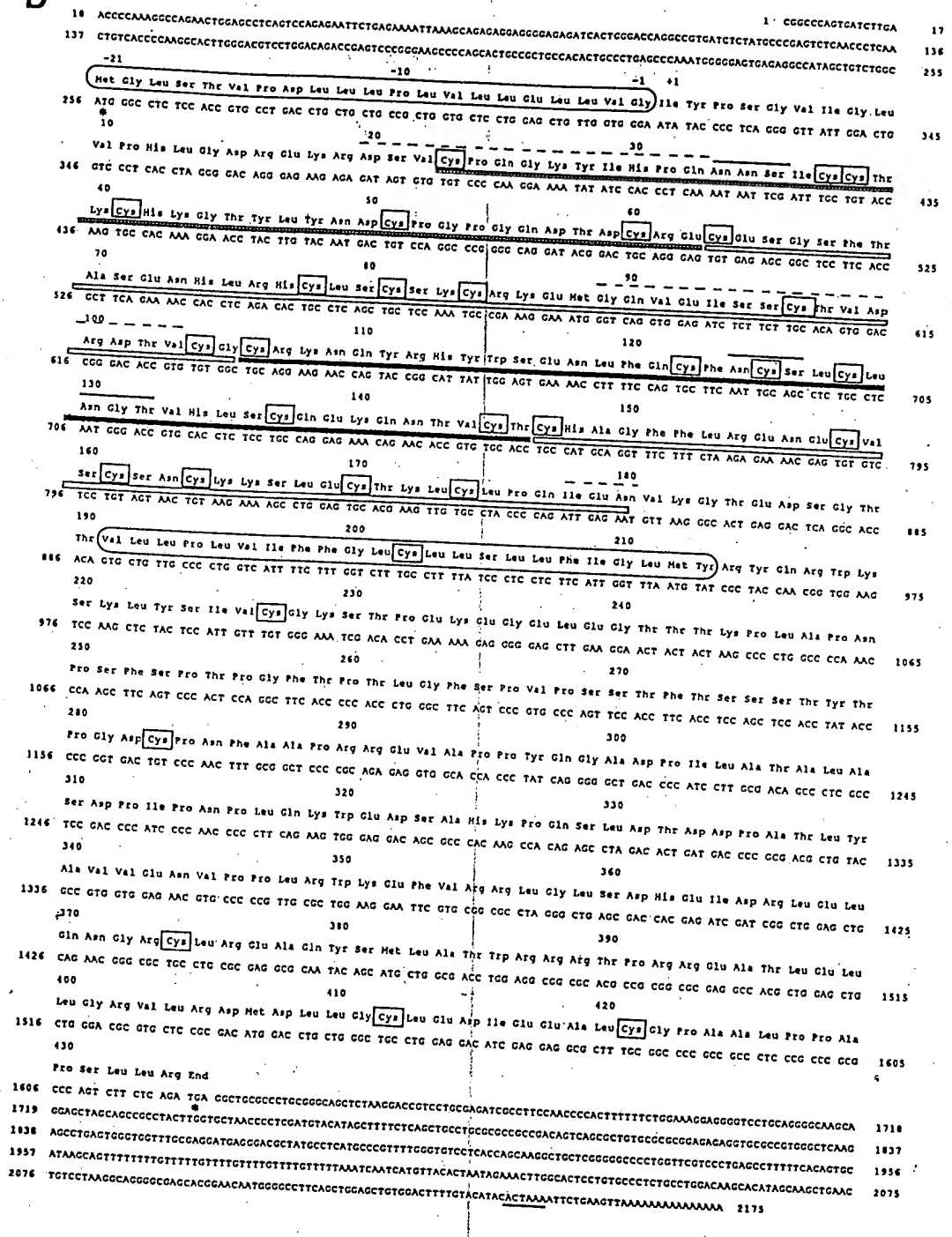

FIGURE 1 D

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Sheet 2 of 8

T/811 A

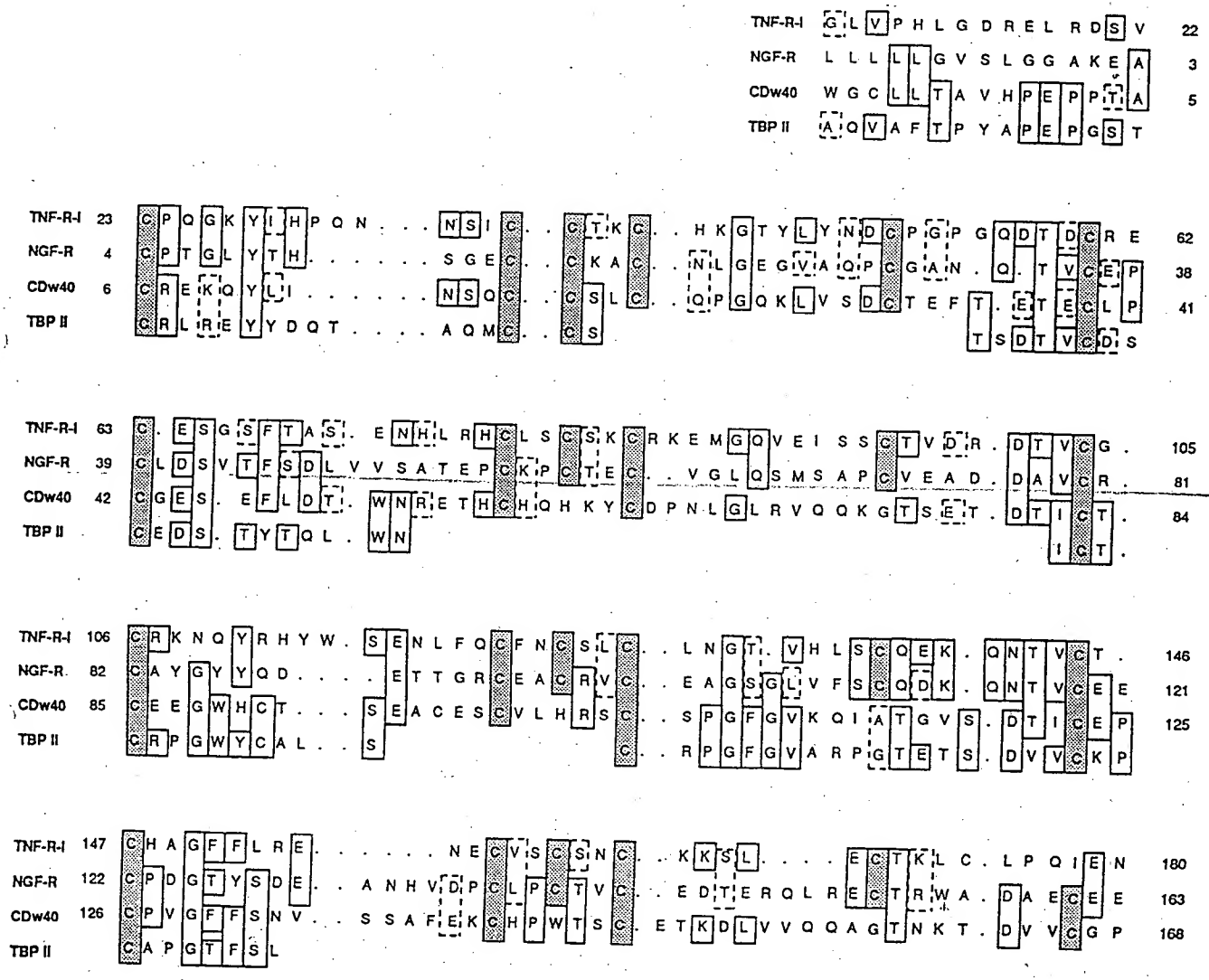

FIGURE 2

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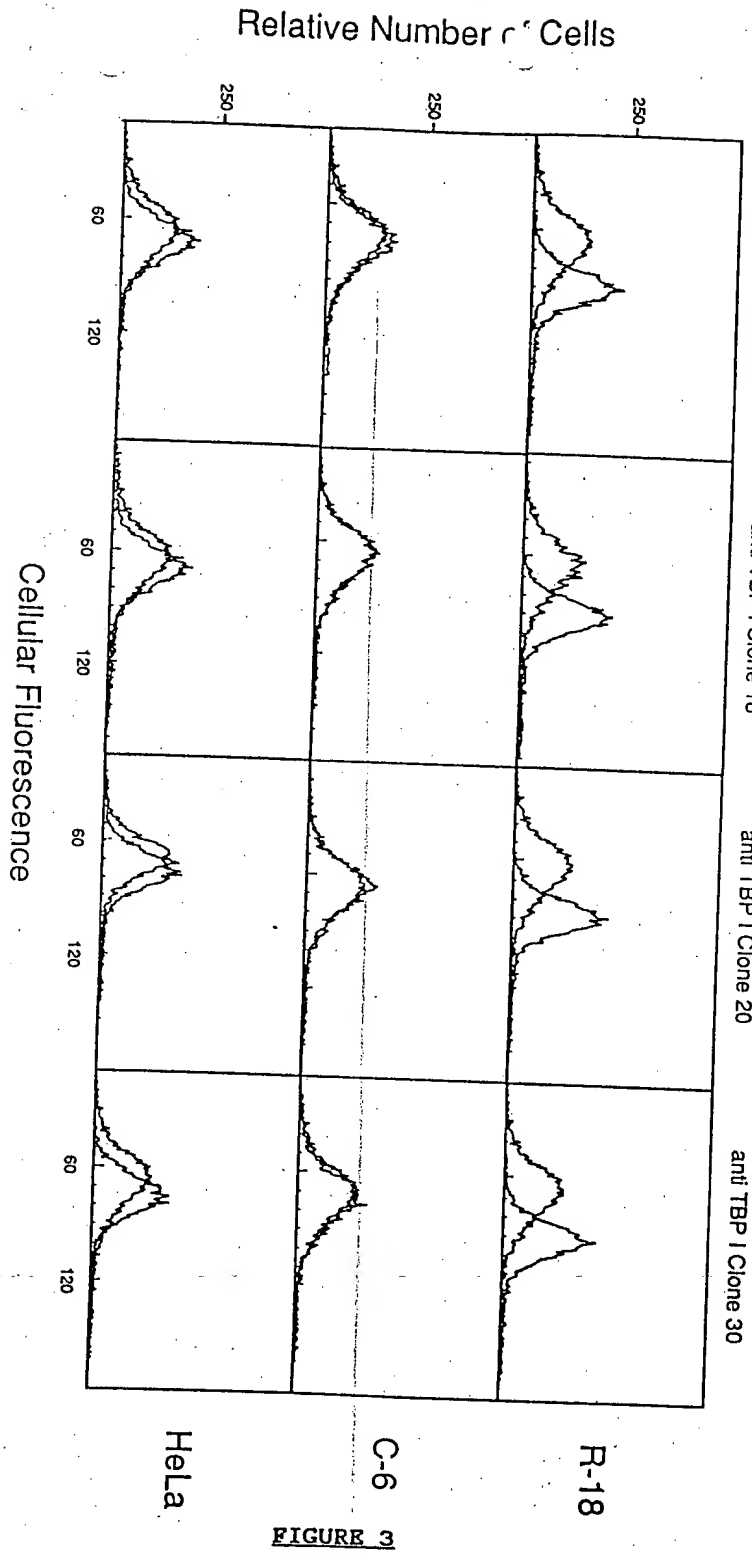
Relative Number of Cells
250
250
250
anti TBP I Clone 17
anti TBP I Clone 18
anti TBP I Clone 20
anti TBP I Clone 30
60
120
Cellular Fluorescence
HeLa
C-6
R-18

FIGURE 3

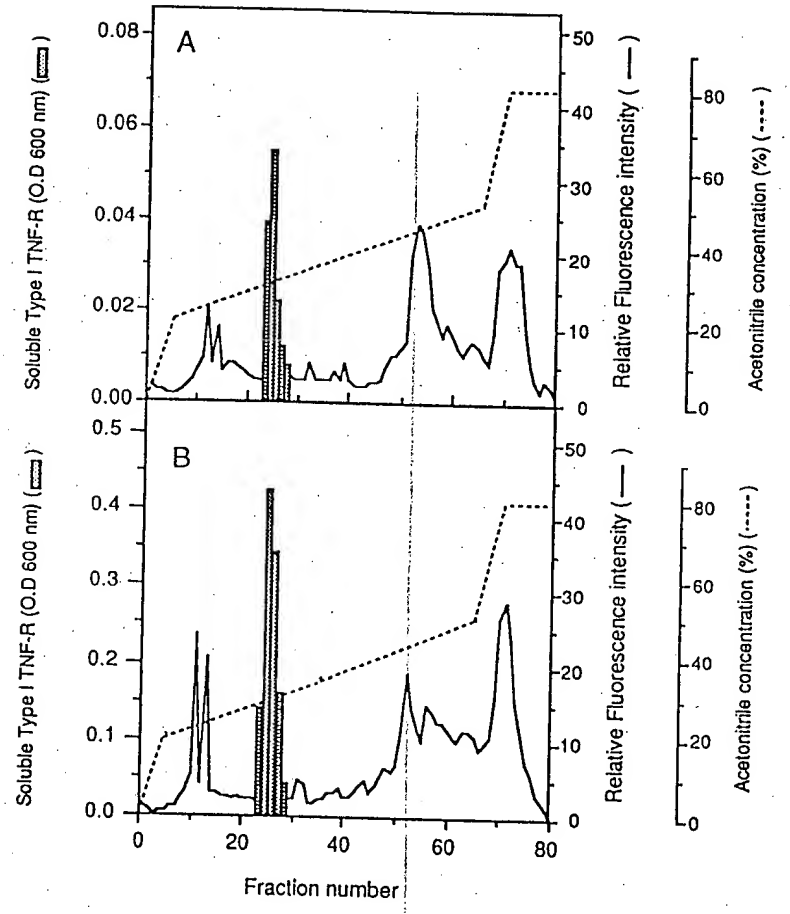

FIGURE 4

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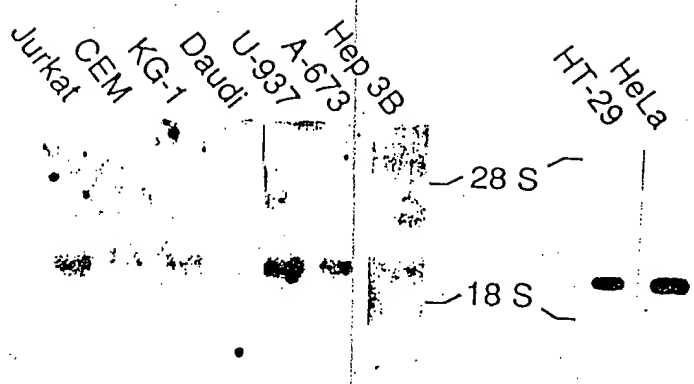

FIGURE 5

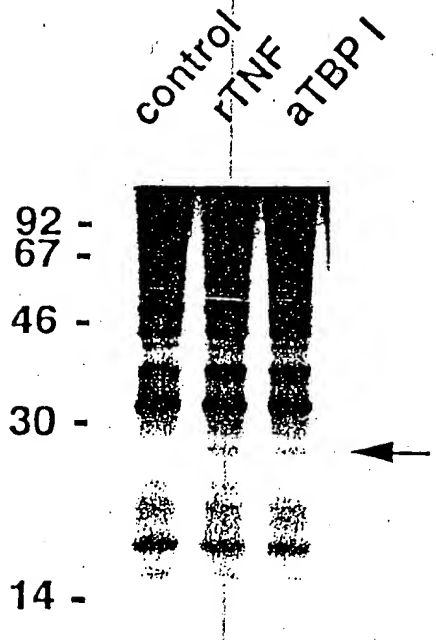

FIGURE 6

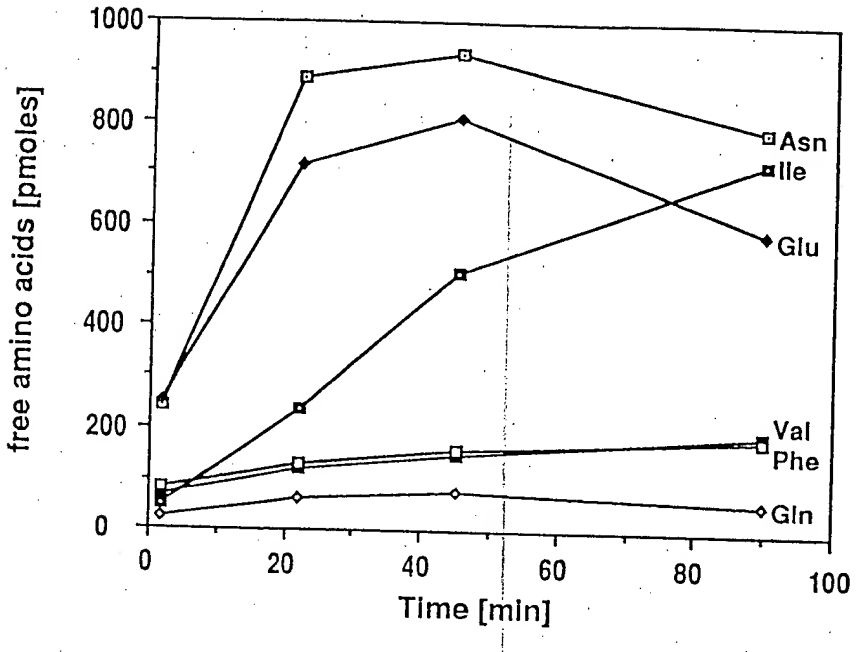

FIGURE 7

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